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BRIEF REPORT

The Role of Rare Protein-Coding Variants in Anti–Tumor Necrosis Factor Treatment Response in Rheumatoid Arthritis

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Objective. **In many rheumatoid arthritis (RA) patients, disease is controlled with anti–tumor necrosis factor (anti-TNF) biologic therapies. However, in a significant number of patients, the disease fails to respond to anti-TNF therapy. We undertook the present study to examine the hypothesis that rare and low-frequency genetic variants might influence response to anti-TNF treatment.**

Methods. **We sequenced the coding region of 750 genes in 1,094 RA patients of European ancestry who were treated with anti-TNF. After quality control, 690 genes were included in the analysis. We applied single-variant association and gene-based association tests to identify variants associated with anti-TNF treatment response. In addition, given the key mechanistic role of TNF, we performed gene set analyses of 27 TNF pathway genes.**

Results. **We identified 14,420 functional variants, of which 6,934 were predicted as nonsynonymous 2,136 of which were further predicted to be "damaging." Despite the fact that the study was well powered, no single variant or gene showed study-wide significant association with change in the outcome measures disease activity or European League Against Rheumatism response. Intriguingly, we observed 3 genes, of 27 with nominal signals of association ($P < 0.05$), that were involved in the TNF signaling pathway. However, when we performed a rigorous gene set enrichment analysis based on association *P* value ranking, we observed no evidence of enrichment of association at genes involved in the TNF pathway ($P_{enrichment} = 0.15$, based on phenotype permutations).**

Conclusion. **Our findings suggest that rare and low-frequency protein-coding variants in TNF signaling**

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pathway genes or other genes do not contribute substantially to anti-TNF treatment response in patients with RA.

Rheumatoid arthritis (RA) is effectively managed in many cases with therapies that block the inflammatory cytokine tumor necrosis factor (TNF) (1). However, in approximately one-third of patients, the disease fails to respond to anti-TNF therapy (1). The biologic mechanisms underlying treatment failure are unknown, which limits the development of biomarkers to guide anti-TNF use in the clinical setting. To define genetic predictors of anti-TNF response, investigators have conducted several genome-wide association studies (GWAS) (2–5). To date, few studies have demonstrated significant associations between common genetic variants and anti-TNF response, and no loci have consistently been replicated across studies.

Importantly, genetic studies for anti-TNF response have not investigated rare or low-frequency variants because these were not included on previous versions of genotyping arrays or were excluded in the analysis. These variants are expected to be under purifying selection and thus are potentially enriched for deleterious, protein-coding mutations (6) that may have large effects.

We hypothesized that these rare and low-frequency variants in relevant genes might influence response to anti-TNF treatment. Herein we report on a rare-variant study with anti-TNF treatment response data collected through an international collaboration in which rare and low-frequency variants in 750 genes from 1,094 anti-TNF–treated RA patients were examined. Our primary outcome measure was change in the 28-joint Disease Activity Score (DAS28) (7) from baseline to 3–12 months after initiation of therapy. We performed single-variant and gene-based analysis of the association between rare/low-frequency variants and anti-TNF treatment response.

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PATIENTS AND METHODS

Samples and clinical data. All patients met the American College of Rheumatology 1987 criteria for RA (8) and/or were diagnosed by a board-certified rheumatologist. Written informed consent was provided by all patients, and institutional review board approval was obtained at all sites. To be enrolled, patients had to have at least moderate disease activity (DAS28 >3.2) at the initial time point. We enrolled patients from 8 cohorts in 5 countries: 1) the Autoimmune Biomarkers Collaborative Network (ABCoN) (US; n = 31), 2) the Genetics Network Rheumatology Amsterdam (n = 11), 3) the BeSt study (Dutch Behandelstrategieën voor Rheumatoide Arthritis) (n = 46), 4) the Biologics in Rheumatoid Arthritis Genetics and Genomics Study Syndicate (BRAGGSS) (UK; n = 76), 5) the Dutch Rheumatoid Arthritis Monitoring registry (DREAM) (n = 189), 6) Research in Active Rheumatoid Arthritis (ReAct) (France; n = 294), 7) the Consortium of Rheumatology Researchers of North America (US; n = 87), and 8) the Rheumatic Diseases Portuguese Register (Reuma.pt) from the Portuguese Society of Rheumatology (n = 360).

The following clinical data were collected in each cohort: 1) DAS28 at baseline, 2) DAS28 from at least 1 subsequent time point, usually 3–6 months after initiation of anti-TNF therapy, 3) sex, 4) age, 5) concurrent methotrexate use, and 6) autoantibody status (rheumatoid factor or anti–cyclic citrullinated peptide). We assessed disease activity according to the DAS28; the DAS28 using the C-reactive protein level was used in the ABCoN cohort and the DAS28 using the erythrocyte sedimentation rate (ESR) in the others. Our primary outcome measure was change in the DAS28 from baseline (ΔDAS28, i.e., baseline DAS28–ending DAS28). Responder status according to the European League Against Rheumatism (EULAR) criteria (9) using baseline and ending DAS28 was a secondary outcome measure, excluding the moderate responder category based on the hypothesis that analysis of more extreme phenotypes (good responders versus non-responders) would yield improved discrimination. We examined clinical variables for association with the primary and secondary outcome measures using multivariate linear and logistic regression, respectively. Age, baseline DAS28, concomitant methotrexate therapy, and patient cohort were strongly associated with ΔDAS28. As a result, we included these variables, together with sex, as covariates in all subsequent genetic association tests (see Supplementary Table 1, on the *Arthritis & Rheumatology* web site at http://onlinelibrary.wiley.com/doi/10.1002/art.39966/abstract). Principal components (calculated as described below) were not associated with ΔDAS28, and were not included as covariates in the analysis.

Targeted exon sequencing. We sequenced exons targeted from 828 genes, together with all reported noncoding RA GWAS single-nucleotide polymorphisms (SNPs) and noncoding regions overlapping histone modification marks in CD4+ T cells (which were not analyzed in the present study). In total we sequenced ~2 Mb coding and noncoding sequences. We included genes in known RA risk loci or associated with other autoimmune diseases, immunodeficiency genes, genes identified from mouse models of inflammatory arthritis, and genes in the TNF signaling pathway.

DNA libraries from individual patients were sequenced by pooling 96 libraries after bar-coding. Target genomic regions were enriched using NimbleGen Sequence Capture technology. After target capture, we loaded each pool into 2 lanes of a HiSeq

sequencer. We aligned reads to the reference human genome (NCBI Build37/hg19) using Burrows-Wheeler Aligner and removed duplicate reads using Picard. In total, 95% of the samples reached a minimum average coverage of 20× in >70% of target regions, with 96% of the target regions in the samples passing this initial quality control covered at ≥20× coverage. Sequencing, initial quality control, and SNP calling were performed at The Genome Institute. To account for population stratification, we used 138 ancestry-informative markers targeted for sequencing and passing quality control to calculate principal components using EigenSoft version 4.2 (10), with HapMap phase 3 samples as reference populations. After applying stringent filters, we obtained a final set of 1,094 cases of European ancestry and restricted our analysis to 750 genes with high coverage across the coding sequence.

SNPs were called with Samtools version 1.16 and VarScan 2.2.9, using stringent minimum coverage, mapping quality, and strandness filters. We merged SNP calls from each sample by using both calling algorithms and filtered variants, further removing variants with missingness of >0.05 and a Hardy-Weinberg equilibrium P value of $<10^{-7}$. Finally, we included only variants passing filters in >50% of the samples in the subsequent analysis. Variants were identified at 1 SNP/97 bp density; the transition:transversion ratio based on the variants passing quality control was 3.17. We used AnnoVar to annotate the variants. Variants were then grouped as synonymous or nonsynonymous. The function of missense variants was predicted using PolyPhen-2 and Sift. Variants were recorded as "damaging" if classified as possibly or probably damaging with both PolyPhen-2 and Sift. We also included the nonsense and splice variants in the "damaging" variants group.

Association analysis. We first tested the association of each common variant (minor allele frequency [MAF] ≥1%) with the primary outcome measure (ΔDAS28) and EULAR good response versus no response using a linear regression model and logistic model adjusted for covariates in Plink. We also conducted gene-based association tests to investigate the contribution of rare variants (defined as MAF <1%) to anti-TNF treatment response. This analysis was restricted to 631 genes with at least 2 different rare variants. We used a simpler method that 1) collapses rare variants per gene to identify carriers and noncarriers of rare variants and 2) performs a linear regression or logistic regression analysis to test for association between rare variant carrier status and ΔDAS28 or EULAR good response versus no response. This method entails the assumption that rare variants have a shared direction of effect on the phenotype. We investigated the contribution of 1) all variants in the coding region including synonymous and nonsynonymous rare variants, 2) nonsynonymous rare variants, and 3) a subset of nonsynonymous variants that were predicted to be damaging. For each test performed, we adjusted for covariates, and performed ≥1,000 permutations of the phenotype residuals to calculate empirical P values. As a sensitivity analysis we used the Skat-O method, which can include continuous outcome measures as well as adjust for other covariates, and allows for variants to have opposite effects, to assess association between genes and ΔDAS28 as a sensitivity analysis. Study-wide significance was defined using the Bonferroni method. A significance level of $P < 2.6 \times 10^{-5}$ was used for common variants analysis based on 1,908 tests, and $P < 7.9 \times 10^{-5}$ was used for gene-based association analysis based on 631 tests. We also applied the false discovery rate (FDR) method for multiple testing adjustments.

Data on DAS28 components in 714 patients from the ABCoN, BRAGGSS, DREAM, ReAct, andReuma.pt cohorts were collected. We performed a secondary gene-based association analysis on rare coding variants with more objective DAS28 components, i.e., ESR and swollen joint count (SJC). We used Δlog-transformed ESR, and Δlog-transformed SJC as outcomes, adding 1 to all SJCs to avoid values of 0.

Gene set enrichment analysis. To assess the enrichment of association of rare variants in genes from the TNF signaling pathway, we performed a gene set enrichment analysis (GSEA) using the Kolmogorov-Smirmov test. The goal of GSEA is to determine whether P values are randomly distributed or whether the P values of a given subgroup of sequenced genes are enriched for significant P values compared to the other genes tested. The advantage of GSEA is its relative robustness to outliers.

RESULTS

Findings of targeted exon sequencing in the RA patients. We targeted 828 genes for exon sequencing in 1,383 RA patients of European ancestry who had received anti-TNF treatment. After stringent quality control of the sequencing data, 1,094 RA patients were included in subsequent analyses. Details of the sample collections, as well as clinical data, are shown in Table 1. We restricted our analysis to 750 genes with high coverage across the coding sequence. Among the 14,420 variants identified in these 750 genes, the largest proportion of observed variants was intronic (49.8%), and ~15% of the variants were annotated in functional domains.

Single SNP association analysis results. We first tested 1,908 common variants (MAF ≥1%) individually for association with the primary outcome measure (ΔDAS28), by linear regression analysis. None of these variants reached study-wide significance ($P < 2.6 \times 10^{-5}$) (see Supplementary Table 2, on the *Arthritis & Rheumatology* web site at http://onlinelibrary.wiley.com/doi/10.1002/art.39966/abstract). We also tested for association with the secondary outcome measure (the dichotomized EULAR response) using logistic regression and found no individual variant reaching study-wide significance. In both analyses, all variants showed FDR q values of >0.25 (Supplementary Table 2).

Gene-based association analysis results. We then investigated the contribution of rare protein coding variants (MAF <1%) to treatment response. Among 14,420 variants in coding regions, there were 10,984 rare variants. Of these rare variants, 4,050 were predicted as synonymous and 6,934 were predicted as nonsynonymous, 2,136 of which were predicted to be damaging.

Of the 750 genes with high-quality sequencing data, 631 harbored at least 2 rare protein-coding variants. In these genes, we tested the following for association with ΔDAS28: 1) all coding rare variants, 2) all rare variants predicted to be nonsynonymous, and 3) all nonsynonymous rare variants predicted to be damaging. However, none of the analyses reached study-wide significance ($P < 7.9 \times$

Table 1. Rheumatoid arthritis patient cohorts and clinical data*

	Cohort								
	ABCoN	BeSt	BRAGGSS	CORRONA	DREAM	GENRA	ReAct	Reuma.pt	Total
Sample size									
Total	31	46	76	87	189	11	294	360	1,094
EULAR response									
Good responders	13	30	41	47	105	10	104	109	459
Nonresponders	7	9	28	19	83	1	50	83	280
Clinical variables									
Age, years	55.4±12.8	51.9±14.3	52.1±13.6	59.4±12.8	54.3±12.0	49.5±9.2	54.4±11.2	52.5±12.2	–
Female %	80.7	63	79	75.9	64.6	72.7	76.5	89.2	–
MTX treatment, %	64.5	100	89.5	66.7	76.2	90.9	47.6	82.2	–
Baseline DAS28	5.3±0.7	3.8±0.7	6.3±1.0	4.9±1.2	5.0±1.2	5.4±1.1	5.8±1.0	5.8±1.1	–
ΔDAS28	1.65±1.34	1.60±1.1	2.2±1.9	1.9±1.5	1.5±1.5	3.0±1.2	2.1±1.2	1.8±1.3	–

* Except where indicated otherwise, values are the mean ± SD. ABCoN = Autoimmune Biomarkers Collaborative Network; BeSt = Behandelstrategieën voor Rheumatoide Arthritis; BRAGGSS = Biologics in Rheumatoid Arthritis Genetics and Genomics Study Syndicate; CORRONA = Consortium of Rheumatology Researchers of North America; DREAM = Dutch Rheumatoid Arthritis Monitoring registry; GENRA = Genetics Network Rheumatology Amsterdam; ReAct = Research in Active Rheumatoid Arthritis; Reuma.pt = Rheumatic Diseases Portuguese Register; EULAR = European League Against Rheumatism; MTX = methotrexate; DAS28 = 28-joint Disease Activity Score.

10^{-5}, QQ plot for all rare variants predicted to be nonsynonymous), and all FDR q values were >0.5 (Supplementary Figure 1 and Supplementary Table 3, http://onlinelibrary.wiley.com/doi/10.1002/art.39966/abstract). There were 7, 3, and 7 genes with *P* values of <0.01 (31, 32, and 29 with *P* values of <0.05) when the analyses were restricted to coding variants, nonsynonymous variants, and damaging variants, respectively. Detailed gene-based association results for ΔDAS28 are presented in Supplementary Table 3. In the analysis restricted to nonsynonymous variants, the 3 genes with *P* values of <0.01 were *NFKBIA* ($P = 0.0017$), *AICDA* ($P = 0.0043$), and *CDK6* ($P = 0.0058$). *AICDA* and *NFKBIA* are involved in primary immunodeficiencies, and *NFKBIA* is also involved in the TNF pathway.

When we tested the association between rare variants and TNF blockade response stratified for the 3 major anti-TNF drugs (etanercept, infliximab, and adalimumab), we found no significant associations ($P > 0.0007$) (Supplementary Table 4, http://onlinelibrary.wiley.com/doi/10.1002/art.39966/abstract). Similar results were observed when we examined the secondary outcome measure, EULAR responder versus nonresponder criteria ($P > 0.004$) (Supplementary Tables 3 and 4).

Figure 1. Gene set enrichment analysis for association of tumor necrosis factor signaling pathway genes (defined according to the Kyoto Encyclopedia of Genes and Genomes database) with the primary outcome measure, i.e., change in the 28-joint Disease Activity Score (DAS). *P* for enrichment = 0.15.

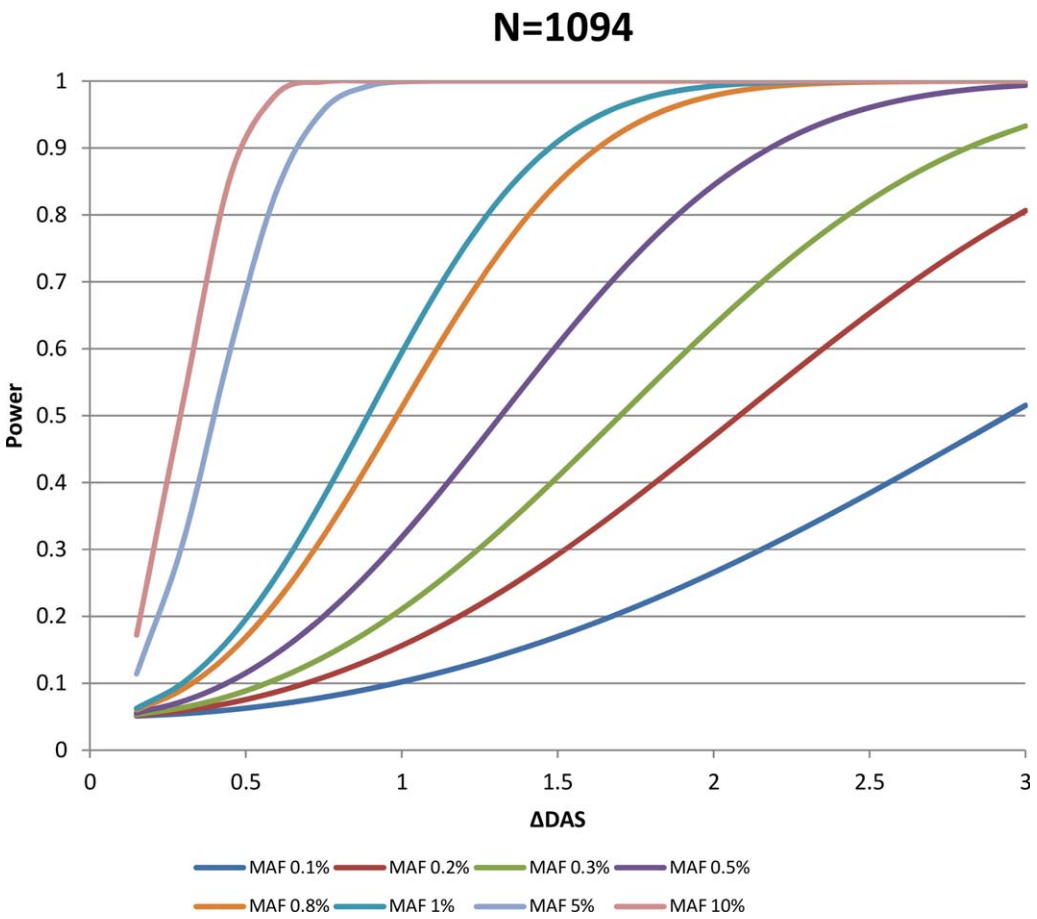

Figure 2. Power to detect an association with the primary outcome measure, i.e., change in the 28-joint Disease Activity Score (DAS), at given effect sizes and minor allele frequency (MAF) based on a sample size of 1,094 patients.

We compared the above association results to those obtained with Skat-O and observed a strong correlation of the *P* values ($R^2 = 0.64$) (Supplementary Figure 2, http://onlinelibrary.wiley.com/doi/10.1002/art.39966/abstract). The main difference was due to genes that exhibited lower *P* values with Skat-O, potentially due to rare variants with opposite effects. Findings of a secondary gene-based analysis of ΔESR and ΔSJC are shown in Supplementary Table 5 (http://onlinelibrary.wiley.com/doi/10.1002/art.39966/abstract); this analysis did not reveal any study-wide significant association.

Gene set enrichment analysis results. Of the 631 genes tested, 27 are involved in the TNF signaling pathway (based on the Kyoto Encyclopedia of Genes and Genomes database). Although none of the TNF pathway genes reached study-wide significance in our gene-based tests, we observed genes from the TNF signaling pathway with *P* values of <0.05, i.e., *NFKBIA* ($P = 0.002$, $\beta = -1.38$), *IL6* ($P = 0.02$, $\beta = 0.42$), and *PTGS2* ($P = 0.04$, $\beta = -0.85$) in the nonsynonymous variant analysis. We ranked the 631 genes by their association *P* values with nonsynonymous variants, calculated the mean rank of the 27 genes from the TNF pathway, and compared this value to the mean rank of the remaining 604 genes. Genes in the TNF pathway were not found to be enriched for rare variants associated with treatment response, compared to the remaining targeted genes ($P_{enrichment} = 0.15$) (Figure 1).

DISCUSSION

The present investigation is, to our knowledge, the largest high-coverage exon sequencing study of anti-TNF–treated RA patients reported to date. Overall, we found little evidence that rare coding variants contribute to anti-TNF response.

To investigate the contribution of rare protein-coding variants to anti-TNF treatment response, we selected a comprehensive list of candidate genes for exon sequencing. Compared to exome chip array analysis, exon sequencing ensures comprehensive capture of rare variants

and allows for more targeted investigation of variants in coding regions. We sequenced up to 50 candidate genes from the top anti-TNF GWAS hits, even though these loci did not reach genome-wide significance, together with genes from the TNF signaling pathway. We also sequenced genes from RA risk loci and candidate genes related to RA or other immune-mediated pathways, under the hypothesis that variants in these genes could also influence response to anti-TNF therapy. As an example illustrating a potential connection between disease risk and treatment response, *TCF7L2* has been shown to be a risk locus for diabetes in association studies, and clear evidence of its association with treatment response in diabetes has been reported (11). Using these gene selection criteria, we tested nearly 10% of genes in the human genome. However, we recognize the limitations of candidate gene studies, many of which have tested candidates that were subsequently shown not to be associated with the phenotype of interest. It remains possible that rare genetic variants within other genes not queried in this study, or in regulatory regions not examined in this study, might still contribute significantly to anti-TNF response.

Expanding association studies to investigate anti-TNF response presents several challenges. While recent anti-TNF genetic studies (12,13), including the present study, include >1,000 RA cases collected from international efforts, the sample sizes remain relatively small compared to other disease cohorts and limit the statistical power to detect modest effect sizes, especially if the MAF is low.

The power to detect an association with ΔDAS28 at different levels of effect size, based on our sample size of 1,094, is plotted in Figure 2. Our study had substantial power to detect clinically relevant single variants with large effect. However, the power for detection of single rare variants with more moderate effect is limited. For instance, for a variant with an MAF of 0.1% there was only ~50% power to detect an association with an effect size of 2 (which corresponds to ΔDAS28 of 3). In contrast, for MAF 0.5% there was >80% power to detect an effect size of 1.3 (ΔDAS28 ~2). We recognize that the power of the study was limited by sample size and the low MAF. It is therefore not surprising that no single variant we tested achieved the study-wise significance level. Despite our efforts to enhance power by using collapsing methods, we did not demonstrate any study-wide significant association in our gene-based association tests. We did observe suggestive associations ($P < 0.05$) in the analysis restricted to nonsynonymous variants, with the top signal mapping *NFKBIA* (NF-κB inhibitor α), with a *P* value of 0.002 ($\beta = -1.38$), driven by 7 rare nonsynonymous variants (Supplementary Figure 1, on the *Arthritis & Rheumatology* web site at http://onlinelibrary.wiley.com/doi/10.1002/art.39966/abstract). We found that *NFKBIA* rare variant carriers had smaller ΔDAS28 (i.e., worse response), corresponding to an effect size of 0.9; nonetheless, given the sample size, this association did not reach the study-wise significance level.

In this study we used ΔDAS28 as an objective marker of anti-TNF treatment response, but perhaps other molecular correlates of treatment response might have been more effective. It has been reported that patient global assessment on a visual analog scale (VAS) and tender joint count subcomponents of the DAS28 are more correlated with psychological variables (14) and less correlated with imaging scores of synovitis (15). We did test the association with specific DAS28 components in our secondary analysis, choosing ΔESR and ΔSJC as outcome measures because these 2 measurements are more objective than tender joint count and patient global assessment on a VAS. We did not observe any evidence of association of either the ESR or the SJC with any of the genes tested. Nonetheless, ΔDAS28 remains the outcome that is used clinically to make decisions regarding continuation (or discontinuation) of therapies, and is therefore an important outcome measure to test for association.

In conclusion, we did not find evidence that rare protein-coding variants in a large set of candidate genes, including genes from the TNF signaling pathways, contribute substantially to anti-TNF treatment response in patients with RA. The identification of molecular biomarkers for treatment response is hence an important goal for future study.

AUTHOR CONTRIBUTIONS

All authors were involved in drafting the article or revising it critically for important intellectual content, and all authors approved the final version to be published. Dr. Cui had full access to all of the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

Study conception and design. Cui, Diogo, Stahl, O'Laughlin, Plenge, Raychaudhuri.

Acquisition of data. Canhao, Mariette, Greenberg, Pappas, Tak, Nurmohamed, Lee, Kurreeman, van der Horst-Bruinsma, Wolbink, Gregersen, Kremer, Crusius, de Vries, Huizinga, Fonseca, Miceli-Richard, Coenen, Barton.

Analysis and interpretation of data. Cui, Diogo, Stahl, Canhao, Mariette, Greenberg, Okada, Pappas, R. S. Fulton, Tak, Nurmohamed, Lee, Larson, Kurreeman, Deluca, O'Laughlin, Fronick, L. L. Fulton, Mardis, van der Horst-Bruinsma, Wolbink, Gregersen, Kremer, Crusius, de Vries, Huizinga, Fonseca, Miceli-Richard, Karlson, Coenen, Barton, Plenge, Raychaudhuri.

ADDITIONAL DISCLOSURES

Authors Diogo and Plenge are currently employed by Merck & Company. Author Tak is currently employed by GlaxoSmithKline.

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